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Dual BRD4 and AURKA Inhibition Is Synergistic against MYCN-Amplified and Nonamplified Neuroblastoma[1]

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Abstract

A majority of cases of high-risk neuroblastoma, an embryonal childhood cancer, are driven by MYC or MYCN-driven oncogenic signaling. While considered to be directly "undruggable" therapeutically, MYC and MYCN can be repressed transcriptionally by inhibition of Bromodomain-containing protein 4 (BRD4) or destabilized post-translationally by inhibition of Aurora Kinase A (AURKA). Preclinical and early-phase clinical studies of BRD4 and AURKA inhibitors, however, show limited efficacy against neuroblastoma when used alone. We report our studies on the concomitant use of the BRD4 inhibitor I-BET151 and AURKA inhibitor alisertib. We show that, *in vitro*, the drugs act synergistically to inhibit viability in four models of high-risk neuroblastoma. We demonstrate that this synergy is driven, in part, by the ability of I-BET151 to mitigate reflexive upregulation of AURKA, MYC, and MYCN in response to AURKA inhibition. We then demonstrate that I-BET151 and alisertib are effective in prolonging survival in four xenograft neuroblastoma models *in vivo*, and this efficacy is augmented by the addition of the antitubule chemotherapeutic vincristine. These data suggest that epigenetic and posttranslational inhibition of MYC/MYCN-driven pathways may have significant clinical efficacy against neuroblastoma.

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Introduction

Advanced neuroblastoma, the embryonal childhood cancer arising from sympathoadrenal precursors, remains a major clinical challenge. Patients with high-risk tumors at diagnosis are treated with aggressive multimodal chemotherapies, radiation therapy, and immunotherapy but suffer high rates of disease progression and/or recurrence, with cure rates ~50% [1,2]. Those patients with progressive neuroblastoma rarely have durable responses to current salvage therapies and die of disease [3]. *MYCN* and/or *MYC* amplification or overexpression have been shown to be oncogenic drivers in a majority of advanced neuroblastomas [4–7]. These proteins are transcription factors, promoting expression of numerous oncogenes and enhancing cell proliferation and survival [8], but also function as repressors of cell signaling [9,10] and as drivers of transcriptional elongation and activation of superenhancers through interactions with CDK7/9 and RNA polymerase II [11–13].

MYC and MYCN are difficult to therapeutically target directly, but novel agents have been designed to destabilize or repress these oncoproteins indirectly. One class of drugs against MYC/MYCN-driven cancers targets Aurora Kinase A (AURKA), a protein with multiple functions in cytokinesis [14] and in the stabilization of MYC and MYCN, by prevention of FBXW7-mediated ubiquitination [15].

Abbreviations: AURKA, aurora kinase A; BRD4, Bromodomain-containing protein 4; CI, combination index; F_a, fraction affected; p-AURKA, phosphorylated AURKA, specifically at threonine 288; LOH, loss of heterozygosity; 11q, long arm of chromosome 11.

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The first-in-class drug, alisertib, showed efficacy against neuroblastoma, particularly MYCN-amplified disease, preclinically [16]. However, in the Phase 1 pediatric clinical trial, it had higher toxicity in children than in adults, limiting its maximally tolerated dose [17]. Alisertib failed to meet response criteria in multiple phase 2 studies when used alone [18–21] but is being examined in combination therapies.

A second class of drugs against MYC/MYCN-driven cancers inhibits the bromodomain and extraterminal motif (BET) chromatin-binding proteins. These proteins recognize and localize to acetylated lysine residues [22] and promote transcription by recruiting and phosphorylating components of RNA Polymerase II [23]. One BET protein, BRD4, has been shown to be active in cancers by promoting expression of multiple targets, including *CDK4/6* [24], *BCL2* [25], *MCL1* [26,27], *MYC* [28], and *MYCN* [29]. BRD4 inhibitors, developed for research and clinical use, have shown some preclinical efficacy against *MYCN*-amplified neuroblastoma but did not induce regression when used alone [25,29,30]. The cytostatic effects of BRD4 inhibitors suggest that these drugs may have limited effects clinically when used alone, particularly in diseases where BRD4 supports oncogenesis but is not the primary disease driver.

AURKA and BRD4 inhibitors both attack many common oncogenic drivers in distinct but complementary ways. In this study, we show that the AURKA inhibitor alisertib and the BRD4 inhibitor I-BET151 have significant synergy against neuroblastoma cell lines *in vitro*, inhibiting viability at significantly lower doses than when either drug is used alone. We show that cells treated with alisertib have a reflexive transcriptional upregulation of *AURKA, MYC*, and *MYCN*, but concomitant treatment with I-BET151 represses that upregulation. Treatment with both drugs is more effective at repressing expression of multiple oncoproteins, including MYC, MYCN, CDK4/6, AURKA, and BCL2. In four tumor xenograft models, I-BET151 and alisertib are more efficacious together in extending survival than either drug alone and induce tumor regression in an *MYCN*-amplified model. Furthermore, the addition of the anti-tubulin chemotherapeutic vincristine augments this effect, inducing durable tumor regression that is maintained after cessation of treatment in an *MYCN*-amplified model and an *MYCN*-nonamplified model and extending survival in a third *MYCN*-nonamplified model.

Materials and Methods

Cell Lines

SK-N-SH cell line was obtained from Javed Khan (National Cancer Institute, Bethesda, MD). SK-N-AS cell line was obtained from American Type Culture Collection (Manassas, VA). NB1643 and NB-SD cell lines were obtained from Peter Houghton (Greehey Children's Cancer Research Institute, San Antonio, TX). All cell lines were authenticated by PowerPlex16 short tandem repeat analysis (Promega) at the start of *in vitro* studies and again prior to *in vivo* studies. Cells were cultured in DMEM (Corning, Bedford, MA) with 10% FBS (PeakSerum, Wellington, CO) at 37°C with 5% CO_2 and confirmed to be free of *Mycoplasma* by SouthernBiotech Mycoplasma Detection Kit (Birmingham, AL), tested every 3 months.

Drugs

Alisertib was purchased from ApexBio (Houston, TX). I-BET151 was obtained from GlaxoSmithKline (Collegeville, PA). A list of primers and antibodies used can be found in the supplementary data.

Cell Viability Assay, Combination Index (CI) Analysis, and LIVE/DEAD Assay

NB-1643, SK-N-SH, NB-SD, and SK-N-AS cells were plated in 96-well plates at 25,000; 25,000; 25,000; and 5000 cells/well, respectively, in complete media in triplicate wells for each dose and cultured for 24 hours. Cells were treated with either I-BET151 dissolved in DMSO with concentration from 20 to 8000 nM, alisertib dissolved in ethanol with concentrations from 10 to 1000 nM, both drugs, or vehicle control for 48 hours. Cell viability was measured using the IncuCyte ZOOM live cell imaging system (Essen BioScience, Ann Arbor, MI) to track percent confluence of each well. Percentage confluence as compared to vehicle control was used to calculate treatment effect. IC50 and combination index (CI) values were calculated using Compusyn software (Combosyn, Inc., Paramus, NJ). The cells were also treated with the Invitrogen LIVE/DEAD viability/cytotoxicity assay (ThermoFisher Scientific, Waltham MA) using the manufacturer protocol. In brief, at the experiment end point, medium was removed from the cells and washed with PBS. The cells were then treated with PBS containing 1 μM calcein AM and 2 μM ethidium homodimer. Viable cells take up the calcein AM, and dead cells take up the ethidium homodimer. Cells were incubated for 45 minutes and then imaged using the IncuCyte Zoom with fluorescence imaging settings. Viability was assessed by green fluorescence; cytotoxicity was assessed by red fluorescence. Three independent experiments were performed; representative experiments are shown here.

Reverse Transcription–Quantitative Polymerase Chain Reaction (RT-qPCR)

Cells were grown to 80% confluence and then treated with 1 μM I-BET151, 1 μM alisertib, both drugs at 1 μM, or vehicle control for 24 hours. Total RNA was extracted from the cells using NucleoSpin RNA purification kit (Takara Bio USA), and 1 μg of RNA was used for cDNA synthesis using Maxima RT cDNA First Strand Synthesis kit (ThermoFisher Scientific). qPCR was performed using KiCqStart SYBR Green qPCR ReadyMix (Sigma-Aldrich, St. Louis, MO) using the ABI PRISM 7900HT thermal cycler (ThermoFisher Scientific), with relative quantitation by the ddC_t method as previously described [31]. Experiments were performed with technical duplicates on each plate and in three independent experiments, with the relative expression of each experiment used to calculate expression and standard deviation, plotted on each graph.

Western Blot

Cells were grown to 80% confluence and then treated with 1 μM I-BET151, 1 μM alisertib, both drugs at 1 μM, or vehicle control for 48 hours. Cells were collected by scraping and lysed using RIPA buffer, with 50 μg of protein/sample used for Western blot as previously described [31]. Blots were imaged by chemiluminescence using ECL Western Blotting Substrate (ThermoFisher Scientific). Band intensity was quantified using ImageQuant TL software (GE Healthcare, Marlborough, MA) and then normalized by comparing each band to its actin control sample then to the vehicle control sample to generate a ratio of relative expression. Experiments were performed in independent triplicate; representative images are shown here. Complete blots are shown in the Supplementary Data.

Tumor Xenograft Studies

A total of 5×10^6 cells of each type were suspended in PBS and mixed 1:1 in Matrigel (Corning) to a final volume of 100 μl and injected subcutaneously into the flanks of SCID mice (Envigo,